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Hierarchy among Multiple H-2^b-Restricted Cytotoxic T-Lymphocyte Epitopes within Simian Virus 40 T Antigen

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Simian virus 40 large tumor (T) antigen contains three H-2D^b-restricted (I, II/III, and V) and one H-2K^b-restricted (IV) cytotoxic T lymphocyte (CTL) epitopes. We demonstrate that a hierarchy exists among these CTL epitopes, since vigorous CTL responses against epitopes I, II/III, and IV are detected following immunization of *H-2^b* mice with syngeneic, T-antigen-expressing cells. By contrast, a weak CTL response against the H-2D^b-restricted epitope V was detected only following immunization of *H-2^b* mice with epitope loss variant B6/K-3,1,4 cells, which have lost expression of CTL epitopes I, II/III, and IV. Limiting-dilution analysis confirmed that the lack of epitope V-specific CTL activity in bulk culture splenocytes correlated with inefficient expansion and priming of epitope V-specific CTL precursors in vivo. We examined whether defined genetic alterations of T antigen might improve processing and presentation of epitope V to the epitope V-specific CTL clone Y-5 in vitro and/or overcome the recessive nature of epitope V in vivo. Deletion of the H-2D^b-restricted epitopes I and II/III from T antigen did not increase target cell lysis by epitope V-specific CTL clones in vitro. The amino acid sequence SMIKNLEYM, which specifies an optimized H-2D^b binding motif and was found to induce CTL in *H-2^b* mice, did not further reduce epitope V presentation in vitro when inserted within T antigen. Epitope V-containing T-antigen derivatives which retained epitopes I and II/III or epitope IV did not induce epitope V-specific CTL in vivo. T-antigen derivatives in which epitope V replaced epitope I failed to induce epitope V-specific CTL. Recognition of epitope V–H-2D^b complexes by multiple independently derived epitope V-specific CTL clones was rapidly and dramatically reduced by incubation of target cells in the presence of brefeldin A compared with the recognition of the other T-antigen CTL epitopes by epitope specific CTL, suggesting that the epitope V–H-2D^b complexes either are labile or are present at the cell surface at reduced levels. Our results suggest that processing and presentation of epitope V is not dramatically altered (reduced) by the presence of immunodominant CTL epitopes in T antigen and that the immunorecessive nature of epitope V is not determined by amino acids which flank its native location within simian virus 40 T antigen.

CD8$^+$ cytotoxic T lymphocytes (CTL) recognize epitopes in the form of short peptides, usually consisting of 8 to 10 residues, which are presented by major histocompatibility (MHC) class I molecules (for reviews, see references 20, 27, 59, and 82). Peptide ligands presented in MHC class I protein complexes are largely generated by degradation of proteins within the cytosolic compartment (82, 87) and are translocated into the endoplasmic reticulum by a TAP1/TAP2-dependent peptide transporter (5, 36, 39, 57, 68, 88), where they can be assembled into complexes with nascent MHC class I heavy-chain and β_2-microglobulin subunits (46, 67, 83, 84). It is unclear whether epitopes released from viral or self proteins are shortened to the final (optimal) epitope length prior to transport. For example, the TAP1/TAP2 transporter can transport peptides which are longer than required for optimal T-cell recognition, and all proteolytic trimming (processing) events required for the formation of epitope peptides need not be completed in the cytoplasm (3, 18, 53, 65, 66). Discrimination among peptides of similar lengths which differ in primary sequence may occur at the level of TAP-dependent peptide translocation (49, 52, 53). The mechanism(s) responsible for directing assembly of peptide-MHC class I complexes in the endoplasmic reticulum remains unresolved but may include direct interaction between TAP and class I molecules (4, 55, 70). Efficient formation of peptide-MHC class I complexes appears to require that potential peptide ligands contain amino acid residues which can be accommodated by corresponding pockets in a cleft formed between the α1 and α2 domains of the class I heavy chain (7, 24, 48, 89). Pool sequencing of native peptides eluted from MHC class I complexes has revealed allele-specific peptide sequence motifs which agree well with epitope sequences inferred by other methods (21, 59). Appropriately assembled MHC class I-peptide complexes are released from the endoplasmic reticulum and access the cell surface via an exocytic pathway (16, 27, 32).

Why all amino acid sequences which occur within a foreign (non-self) protein possess appropriate MHC class I binding motifs and bind efficiently to MHC class I complexes are not immunogenic remains unclear. Failure to induce a CTL response does not necessarily result from an absence of potentially responsive T cells in the repertoire. Several studies have shown that protein sequences which immediately flank an epitope can affect the efficiency with which it is presented on the cell surface in functional MHC class I complexes (6, 17, 19, 30). It is clear from other reports, however, that altering the protein context from which an epitope is expressed need not inhibit recognition by cognate epitope-specific CTL (17, 25, 29). Moreover, protein context effects which result in reduced efficiency of CTL recognition in vitro may or may not correlate

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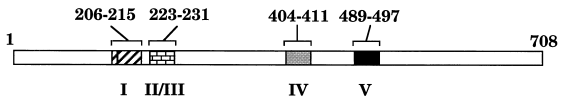

Epitope	Location	Residues	Restriction	CTL Clones	Reference(s)
I	206-215	SAINNYAQKL	H-2D^b	Y-1	42, 72
				K-11	9
II/III	223-231	CKGVNKEYL	H-2D^b	Y-2	15, 72
				Y-3	72
				K-19	9
IV	404-411	VVYDFLKC	H-2K^b	Y-4	50, 72
V	489-497	QGINNLDNL	H-2D^b	Y-5	15, 71
				H-1	this study
				B-3	this study

FIG. 1. H-2^b-restricted CTL epitopes in SV40 T-antigen and SV40-specific CTL clones. H-2^b-restricted CTL epitopes in SV40 T antigen are indicated by the filled boxes (I, II/III, IV, and V). Amino acid positions and minimal epitope sequences are indicated for each epitope. MHC restriction and designations are given for cognate CTL clones.

with reduced immunogenicity in vivo (6, 17). No general rule has emerged which can be used to predict whether an amino acid sequence, which minimally conforms to an MHC class I binding motif and is located within a non-self protein, will be efficiently processed, presented to, and recognized by CTL or will be immunogenic in animals of the appropriate MHC haplotype.

The simian virus 40 (SV40) large tumor (T) antigen provides a model system with which to address some of the questions regarding processing and presentation of MHC class I-restricted CTL epitopes. The SV40 T antigen mediates transformation of a variety of mammalian cell types in culture and is involved in induction of neoplasia in vivo (22, 40, 43). T antigen also contains epitopes recognized by MHC class I-restricted CTL generated in *H-2^b* mice upon immunization with purified T-antigen protein, SV40, or cell lines which express T antigen (reviewed in reference 77). CTL clones directed against multiple defined, H-2^b-restricted epitopes within T antigen have been isolated and characterized (9, 71, 72). Immortalized fibroblast cell lines expressing epitope loss variants of T antigen have been generated (73, 74), and the mutations affecting the T-antigen genes expressed by these CTL escape variants have been characterized (41, 50). The SV40 T antigen provides a useful tool for probing factors which affect presentation of epitopes to, and recognition by, MHC class I-restricted CTL.

Four distinct H-2^b-restricted CTL recognition sites have been identified and mapped within SV40 T antigen with the use of SV40 T-antigen-specific CTL clones, deletion mutants of T antigen, and panels of synthetic peptides (2, 14, 15, 42, 50, 71, 72). Epitopes I, II/III, and V are H-2D^b restricted and represent epitopes recognized by the CTL clones Y-1 and K-11, Y-2, Y-3 and K-19, and Y-5, respectively. Clone Y-4 is H-2K^b restricted (72) and recognizes epitope IV. The sequences of the epitopes recognized by these CTL clones are given in Fig. 1.

The multiplicity of H-2^b-restricted, SV40-specific CTL raises the question of the immunodominant and immunorecessive nature of these CTL epitopes within T antigen. T-antigen-specific H-2D^b-restricted CTL and H-2K^b-restricted CTL precursors (CTLp) are readily demonstrated following immunization of C57BL/6 (*H-2^b*) mice with syngeneic cells expressing full-length T antigen or appropriate fragments of T antigen (33, 72). Previous results have suggested that not all H-2^b-restricted epitopes induced CTL immune responses of equal magnitude. For example, lytic activity directed against epitope V in T antigen was demonstrated only following inoculation of *H-2^b* mice with the epitope loss variant cell line B6/K-3,1,4, which retains functional expression of only epitope V (71). Moreover, repeated in vitro stimulation of the spleen cells with B6/K-3,1,4 cells was required to generate CTL specific for epitope V. Accordingly, the H-2D^b-restricted epitope V of SV40 T antigen has been characterized as an immunorecessive epitope (71).

The goal of this study was to establish whether a hierarchy exists among the multiple H-2^b-restricted CTL epitopes within SV40 T antigen and to identify factors which may contribute to this hierarchy. In this study, we have examined T-antigen-dependent factors which may affect CTL recognition and/or immunogenicity (ability to induce a detectable CTL response in vivo) of the immunorecessive epitope V (minimally defined as SV40 T-antigen residues 489 to 497). We investigated whether deleting combinations of CTL epitopes I, II/III, and/or IV would lead to altered CTL recognition and/or immunogenicity of epitope V. Furthermore, the contribution of T-antigen protein sequences surrounding epitope V to its immunorecessive nature was evaluated by replacing the immunodominant epitope I with epitope V.

MATERIALS AND METHODS

Plasmids and mutagenesis. Plasmid pSelectESV-1 (50), provided by M. J. Tevethia, The Pennsylvania State University College of Medicine, Hershey, was constructed by inserting a 3.0-kb *Kpn*I-*Bam*HI fragment representing VA45-54 SV40 early-region DNA (encodes large T antigen under control of the SV40 early promoter) into the phagemid pSelect (Promega, Madison, Wis.). Derivatives of T antigen lacking defined H-2^b-restricted CTL epitopes were created by site-directed mutagenesis using pSelectESV-1 (or subsequent Tet^r Amp^s derivatives encoding mutant T-antigen proteins) according to the Altered Sites Mutagenesis procedure (Promega). Double mutants were constructed by sequential rounds of mutagenesis or by simultaneous use of multiple mutagenic oligonucleotides. Oligonucleotides used for mutagenesis and DNA sequencing were synthesized by a MilliGen/Biosearch 7500 DNA synthesizer in the Macromolecular Core Facility at The Pennsylvania State University College of Medicine, Hershey. Codons used to construct T-antigen CTL epitope exchange derivatives were identical to those found in the wild-type VA45-54 coding sequence. The nucleotide sequences of VA45-54 regions which encompass the H-2^b-restricted T-antigen CTL epitopes are identical to those reported previously (81; data not shown). All alterations to T antigen were verified by DNA sequence analysis.

Generation of B6 cell lines expressing SV40 T-antigen epitope deletion and/or epitope relocation derivatives. B6 fibroblast cell lines expressing SV40 T antigen or T-antigen derivatives (Table 1) were obtained following transfection of primary C57BL/6 (*H-2^b* haplotype) mouse embryo fibroblasts with plasmid DNA by the calcium phosphate precipitation method (28) as modified by Wigler et al. (85) and Tevethia (76). Immortalized foci were expanded into continuous cell lines. Cell lines used for cytotoxicity assays were screened by indirect immunofluorescence for nuclear T-antigen expression (37, 80), using monoclonal antibodies which recognize amino-terminal (PAb419) and carboxy-terminal (PAb901) epitopes in T antigen (31, 80). The apparent molecular masses and relative steady-state levels of the T-antigen derivatives produced were compared for selected cell lines by immunoprecipitation from cell extracts followed by Western blotting (immunoblotting) (37; data not shown). Cell lines were maintained in Dulbecco modified Eagle medium supplemented with 5 or 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, 100 μg of kanamycin per ml, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 0.15% (wt/vol) sodium bicarbonate.

Cell lines. Cell lines generated and/or used as part of this study are listed in Table 1 along with a description of the relevant SV40 T antigen. The cell line B6/K-0 was established by transformation of C57BL/6 primary kidney cells with plasmid DNA encoding the wild-type SV40 T antigen from strain VA45-54 (72, 73). The antigen loss variants B6/K-3,1,4 and B6/K-1,4,5 used in this study have been described elsewhere (73, 74); these cell lines were derived by sequential in vitro coculture of B6/K-0 cells with SV40 T-antigen-specific CTL clones. B6/K-3,1,4 cells are resistant to lysis by CTL clones Y-3, Y-2, K-19, Y-1, K-11, and Y-4, while B6/K-1,4,5 cells are resistant to lysis by these same CTL clones as well as Y-5 (51, 73, 74). Mutations within the respective T-antigen genes which affect expression or recognition of the H-2D^b-restricted T-antigen epitopes (I, II/III, IV, and V) in these lines have been determined (41, 50). The B6/WT-3 and B6/WT-19 cell lines were derived by transformation of C57BL/6 mouse embryo

TABLE 1. Cell lines used in this study

Cell line[a]	Transforming agent	SV40 T antigen[b]	Reference(s)
B6/WT-19	SV40	Wild type	58, 78
B6/WT-3	SV40	Wild type	58, 78
B6/K-0	pPVU0	Wild type	72, 73
B6/K-3,1,4[c]		Δ157–254; Tyr-406→His	71, 41, 50
B6/K-1,4,5[c]		Δ134–263; Val-405→Leu; Ile-491→Phe	74, 41, 50
B6/TpLM234-5Aa and -6Bb	pLM234	Wild type	This study
B6/TpLM235-7Ba	pLM235	Δ207–215	This study
B6/TpLM236-8Ab	pLM236	Δ223–231	This study
B6/TpLM237-9Ab	pLM237	Δ404–411	50
B6/TpLM241-11Bb	pLM241	Δ489–497	This study
B6/TpLM247-14Ab and -14Bb	pLM247	Δ207–215; Δ223–231	This study
B6/TpLM249-15Ab and -15Bb	pLM249	Δ207–215; Δ223–231; Δ489–497	This study
B6/TpLM254-18Aa and -18Ba	pLM254	Δ207–215; Δ489–497; 489–497 replace 207–215	This study
B6/TpLM255-19Aa and -19Bb	pLM255	Δ207–215; Δ489–497; 207–215 replace 489–497	This study
B6/TpLM257-20Aa and -20Ba	pLM257	Δ207-215; Δ489–497; 489–497 replace 207–215; 207–215 replace 489–497	This study
B6/TpLM279-32Ab and -32Bb	pLM279	Δ207–215; Δ223–231; Δ489–497; 489–497 replace 207–215	This study
B6/TpLM280-34Ab and -34Ba	pLM280	Δ207–215; Δ223–231; Δ489–497; 207–215 replace 489–497	This study
B6/TpLM281-35Ab and -36Ba	pLM281	Δ207–215; Δ223–231; Δ489–497; 489–497 replace 207–215; 207–215 replace 489–497	This study
B6/TpTR350N5-39A1, -39B1, -40A2, and -40B1	pTR350N5	Residues GISMIKNLEYMANS inserted by using *Eco*RI linker located at residue 350	This study
B6/TpTR350A5-41A1, -41B2, -42A2, and -42B2	pTR350A5	Residues GISMIKALEYMANS inserted by using *Eco*RI linker located at residue 350	This study
B6/TpLM301-59A1, -59A2, -59B1, and -59B2	pLM301	Δ207–215; residues SMIKNLEYM replace 207–215	This study
B6/TpLM323-69A1 and -70A1	pLM323	Δ206–215; Δ489–497; 489–497 replace 206–215; 206–215 replace 489–497	This study
B6/TpLM319-73A1	pLM319	Δ216–222; 489–497 replace 216–222	This study
B6/TpLM325-77A1	pLM325	Δ216–222; Δ489–497; 489–497 replace 216–222	This study
RMA	Rauscher virus	None	35, 44
RMA/s	Rauscher virus	None; *tap2* mutant	35, 44

[a] Groups represent multiple cell lines derived by using the same T-antigen construct.
[b] Numbers correspond to amino acid residue positions in wild-type T antigen. Amino acid residues deleted from T antigen are indicated following the "Δ" symbol. An arrow indicates a single amino acid substitution affecting the corresponding amino acid in T antigen.
[c] B6/K-0 epitope loss variant selected by coculture with SV40-specific CTL clones in vitro.

fibroblasts, using the wild-type SV40 strain VA45-54 (58, 78), and express high levels of T antigen and H-2^b MHC class I antigens. The RMA cell line and the antigen presentation-defective, TAP2-deficient RMA/s cell line have been described elsewhere (35, 44).

Generation and maintenance of SV40-specific CTL clones. Isolation and characterization of the SV40-specific CTL clones Y-1 and K-11, Y-2, Y-3 and K-19, Y-4, and Y-5, which are specific for CTL recognition epitopes I, II/III, IV, and V, respectively, have been described elsewhere (9, 71, 72). Additional site V-specific CTL clones (H-1 and B-3) were generated as described previously (71). The SV40-specific CTL clones are listed in Fig. 1 along with the locations and sequences of the corresponding H-2^b-restricted CTL epitopes in T antigen. Culture conditions used to propagate these CTL clones have been described elsewhere (15, 42, 50). CTL clones were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 25 μg of pyruvic acid per ml, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5 mM HEPES (CTL medium). Clones Y-1, Y-2, Y-3, Y-4, Y-5, H-1, and B-3 were maintained in CTL medium containing 5 to 7.5 U of human recombinant interleukin-2 (AmGen, Thousand Oaks, Calif.) per ml, while clones K-11 and K-19 were cultured in CTL medium containing 10% (vol/vol) T-Stim culture supplement (Collaborative Biomedical Products, Bedford, Mass.) and 50 mM methyl-α-D-mannopyranoside (Sigma Chemical, St. Louis, Mo.). CTL clones were provided with gamma-irradiated (10,000 rad) stimulator cells (1.25×10^5/ml of CTL medium) at each passage. Most CTL clones were stimulated with B6/WT-19 cells. Epitope V-specific CTL clones (Y-5, H-1, and B-3) were stimulated with gamma-irradiated B6/K-3,1,4 cells.

Cytotoxicity assays. Susceptibility of B6 cell lines to lysis by SV40 T-antigen-specific CTL clones was measured in ^{51}Cr release assays as described previously (72, 79). Similar results were obtained whether target cells were labeled with ^{51}Cr before (as a monolayer) or after (in suspension) trypsinization. Unless otherwise indicated, cell lines used in this study were cultured in the presence of gamma interferon (generously provided by H. M. Shepard, Genentech Inc., San Francisco, Calif.) for 2 days prior to their use in cytotoxicity assays (8, 14, 71, 72). Percentage of specific CTL-mediated ^{51}Cr release was calculated by using the formula % specific release = $[(E - S)/(M - S)] \times 100$, where E indicates the radioactivity released from targets incubated in the presence of effector cells, S indicates the spontaneous release of radioactivity by target cells incubated in the presence of medium alone, and M indicates the quantity of radioactivity released into the supernatant by target cells incubated in the presence of 2.5% (wt/vol) sodium dodecyl sulfate. All determinations were made in triplicate. Similar results were obtained in at least two independent assays.

Cytotoxicity assays involving BFA-treated target cells. In some experiments, susceptibility to lysis by CTL clones was assayed following incubation of target cells in the presence of 4 μg of brefeldin A (BFA; Sigma) per ml (12, 86). Typically, appropriate amounts of cells were seeded in T25 flasks on day zero. The medium was replaced on day 1 with 6 ml of fresh medium containing 40 U per ml of gamma interferon. BFA was added to the flasks (from a 1-mg/ml stock solution made up in methanol and stored at −20°C) 10 or 4 h prior to harvest (day 3). Target cells were radiolabeled in the final 2 h prior to harvest by reducing the medium volume to 2 ml and adding 250 μCi of ^{51}Cr. Medium used to wash BFA-treated cells following trypsinization contained 1 μg of BFA per ml. For the cytotoxicity assay, BFA-treated, ^{51}Cr-labeled target cells were suspended in CTL medium containing 2 μg of BFA per ml and plated with an equal volume of effector suspension which lacked BFA.

Synthetic peptides and preparation of peptide pulsed-target cells for use in cytotoxicity assays. Peptides corresponding to sequences within T antigen were synthesized at the Macromolecular Core Facility of The Pennsylvania State University College of Medicine by 9-fluorenylmethoxycarbonyl chemistry, on an automated peptide synthesizer (9050 MilliGen PepSynthesizer). Procedures for preparation and use of peptides in cytotoxicity assays have been described elsewhere (14, 15). ^{51}Cr-labeled target cells were routinely pulsed with peptides by incubation in complete Dulbecco modified Eagle medium or RPMI 1640 medium supplemented with peptide at a concentration of 1 μM. Peptide pulsing was performed either during or after the ^{51}Cr labeling procedure. In either case, peptide-pulsed cells were washed at least twice prior to use in the cytotoxicity assay. Peptides which correspond to amino acid sequences within T antigen are

TABLE 2. Induction of CTL to SV40 T antigen specific for MHC class I-restricted epitopes I, II/III, and IV by immunization of B6/ mice with B6/WT-3 or B6/WT-19 cells

Effectors[a]		E:T ratio[b]	% Specific target cell lysis[c]						
In vivo immunization	In vitro stimulation		B6/WT-3 cells	B6/K-1,4,5 cells pulsed with synthetic peptides					
				LT206–215	LT223–231	LT404–411	LT489–497	No peptide	HSV-1 RR1 822–829
Expt 1									
B6/WT-3	B6/WT-19	50:1	85	62	46	62	2	1	ND
None	B6/WT-19	50:1	4	1	2	3	2	2	ND
Expt 2									
B6/WT-19	B6/WT-19	50:1	ND	68	72	83	5	6	6
		17:1	ND	49	52	68	3	3	4
		6:1	ND	26	30	43	2	1	2
None	B6/WT-19	50:1	ND	7	6	9	6	5	6
		17:1	ND	4	3	6	4	4	5
		6:1	ND	2	2	3	2	2	3

[a] Bulk culture CTL were generated from C57BL/6 mice primed with B6/WT-3 or B6/WT-19 B6 cells and were stimulated in vitro with B6/WT-19 cells. Control animals received either Hanks' balanced salt solution (experiment 1) or were not injected (experiment 2).

[b] E:T, effector-to-target. Effectors were assayed for lytic activity in 4.5- or 5-h ^{51}Cr release assays.

[c] Peptide-pulsed targets were prepared by pulsing B6/K-1,4,5 epitope loss variant cells with peptides representing minimal SV40 CTL epitopes or, as a control, HSV-1 RR1 822-829. Synthetic peptides LT206–215, LT223–231, LT404–411, and LT489–497 correspond to CTL recognition epitopes I, II/III, IV, and V, respectively. ND, not done.

designated by "LT" followed by numbers which indicate the positions within T antigen of the amino acids corresponding to the first and last residues of the synthetic peptide.

Generation of bulk culture CTL for in vitro cytotoxicity assays. Bulk culture CTL were prepared from splenic lymphocytes obtained from groups of four C57BL/6 mice (6 to 12 weeks of age) 14 to 16 days following intraperitoneal injection with 1.5×10^7 to 2.5×10^7 fibroblast cells representing various SV40 T-antigen derivatives. Cell lines were harvested by trypsinization, chilled on ice, and washed once with fresh medium and at least twice with ice-cold Hanks' balanced salt solution. Splenic lymphocytes were prepared as described previously (23, 33, 72) and cultured in 12-well plates (Costar, Cambridge, Mass.). Each well was seeded with 10^7 splenocytes and 5×10^5 gamma-irradiated (10,000 rad) stimulator cells in 4 ml of CTL medium lacking added human recombinant interleukin-2. Cultures were incubated at 37°C in the presence of 5% CO_2 for 5 or 6 days prior to use as effectors in ^{51}Cr release assays.

Determination of CTLp frequency by limiting-dilution analysis. Limiting-dilution cultures were prepared as described previously (33), using splenic lymphocytes isolated from C57BL/6 mice 4 weeks following intraperitoneal immunization with B6/WT-19 cells, which express the wild-type T antigen. Briefly, appropriate numbers of splenic lymphocytes were plated in a volume of 200 μl in the wells of 96-well round-bottom plates in supplemented Iscove's modified Dulbecco modified Eagle medium (33). In addition to immune splenocytes, individual wells received 10^5 gamma-irradiated (2,000 rad) naive spleen cells, 5% (vol/vol) T-Stim culture supplement, 0.1 M methyl-α-D-mannopyranoside, 0.125 U of human recombinant interleukin-2, and 2×10^5 gamma-irradiated (10,000 rad) B6/WT-19 cells as stimulators. Twenty-four wells were prepared, representing each dilution of immune splenocytes (range, 256,000 to 250; serial twofold dilutions), while control wells received only gamma-irradiated splenocytes obtained from naive animals. Cultures were incubated for 7 days at 37°C in a 5% CO_2-humidified atmosphere, after which cells from individual wells were divided and assayed for lytic activity against ^{51}Cr-labeled, peptide-pulsed B6/K-1,4,5 targets. Individual wells were considered to have contained at least one CTLp if the level of ^{51}Cr release exceeded 10% specific lysis. Frequency estimates were made by using the minimal χ^2 method (75).

MHC class I molecule stabilization assay. The relative binding efficiency of each synthetic peptide for MHC class I molecules was determined by measuring the level of MHC class I molecules on the surface of RMA/s cells. The RMA/s cell line is a Rauscher virus-induced T-cell lymphoma which has a low surface expression of MHC class I molecules (35, 44). Increased expression of surface MHC class I molecules can be induced by incubation of RMA/s cells in the presence of haplotype-specific peptide or at low temperature (45, 83, 84). RMA/s cells (3×10^5 to 6×10^5) were incubated in 1 ml of supplemented RPMI medium containing various concentrations of peptides at 37°C for 14 h as described previously (26, 69). Expression of H-2D^b molecules was determined by fluorescence-activated cell sorter (FACS) analysis using an Epics V Flow cytometer/sorter (Coulter Electronics, Inc., Hialeah, Fla.) set at an excitation fluorescence of 500 mW at 488 nm. Cells were washed twice in phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.1% sodium azide (FACS buffer). Cells were then incubated for 45 min on ice with 0.1 ml of the anti-H-2D^b monoclonal antibody 28-14-8 (56). After washing with FACS buffer, the cells were incubated with 0.1 ml of fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 45 min on ice. Cells were washed, fixed in 1% paraformaldehyde in PBS, and analyzed for the level of MHC class I molecule surface expression.

RESULTS

CTL response in *H-2^b* mice to individual SV40 T-antigen epitopes after immunization with a syngeneic SV40 T-antigen-expressing cell line. SV40 T antigen contains three H-2D^b-restricted epitopes (I, II/III, and V) and one H-2K^b-restricted epitope (IV; Fig. 1). The relative contribution of each of these epitopes in the generation of an SV40 T-antigen-specific CD8$^+$ CTL immune response in vivo is not known. To determine if there is a hierarchy among these CTL epitopes, we compared the relative immunogenicities of the H-2^b-restricted epitopes by immunizing B6 mice with B6/WT-3 or B6/WT-19 cells (wild-type T antigen) and stimulating splenic lymphocytes from immunized and control animals with gamma-irradiated T-antigen-expressing B6/WT-19 cells. The bulk culture CTL were assayed for reactivity against the T-antigen CTL epitopes individually by using target cells (B6/K-1,4,5) pulsed with the corresponding synthetic peptides. A peptide corresponding to the herpes simplex virus type 1 ribonucleotide reductase CTL epitope (HSV-1 RR1 822-829 [62]) was used as a negative control. B6/K-1,4,5 cells express a mutant SV40 T antigen which has lost functional expression of CTL epitopes I, II/III, IV, and V recognized by the CTL clones Y-1, Y-2 and Y-3, Y-4, and Y-5, respectively, as a result of deletions or point mutations affecting the respective T-antigen CTL epitopes (41, 50, 73, 74). The results presented in Table 2 show that although CTL specific for epitopes I, II/III, and IV were readily detected by these assays, epitope V-specific CTL were not detected. In the same experiments, the LT489-497 peptide-pulsed B6/K-1,4,5 cells were lysed by the epitope V-specific CTL clone, Y-5 (data not shown).

To confirm that the lack of epitope V-specific cytolytic activity in the bulk culture CTL derived from mice primed with T-antigen-expressing cells resulted from inefficient priming or expansion of epitope V-reactive CTLp, a limiting-dilution analysis was performed. ^{51}Cr-labeled, peptide-pulsed targets were used in this analysis so that the frequencies of CTLp

TABLE 3. Relative frequencies of CTL precursors directed against three H-2^b-restricted CTL recognition epitopes (I, IV, and V) in SV40 T antigen determined by using synthetic peptides[a]

T-antigen epitope	Target peptide[b]	Reciprocal frequency	95% confidence limit
I	LT206–215	67,191	53,335–90,774
IV	LT404–411	14,664	11,670–19,726
V	LT489–497	562,614	394,007–983,475
None	HSV-1 RR1 822-829	316,383	237,418–474,054

[a] Splenic lymphocytes were obtained 4 weeks following intraperitoneal inoculation with B6/WT-19 cells.

[b] Lytic activity was determined in a 5-h cytotoxicity assay in which gamma interferon-induced, ^{51}Cr-labeled B6/K-1,4,5 cells pulsed with synthetic peptides were used as targets.

specific for selected T-antigen CTL epitopes (I, IV, and V) could be measured individually. The results (Table 3) indicated that CTLp specific for epitope IV and, at a reduced frequency, epitope I were readily detected in this assay. By contrast, CTLp specific for targets pulsed with the epitope V peptide LT489-497 were not detected above the frequency of CTLp specific for targets pulsed with a control peptide corresponding to HSV-1 RR1 822-829 (62). In other assays, epitope II/III- and epitope I-specific CTLp were detected at frequencies which were similar to one another but lower than the frequency of epitope IV-specific CTLp (data not shown). These results indicated that epitope V from SV40 T antigen is inefficient at inducing CTL in B6 mice primed with cells expressing the full-length SV40 T antigen and are consistent with our previous characterization of epitope V of SV40 T antigen as an immunorecessive or subdominant CTL epitope (71, 77).

Effect of deleting H-2^b-restricted immunodominant CTL epitopes from SV40 T antigen on the presentation of epitope V by H-2D^b molecules. As a first approach to investigating T-antigen-dependent factors which might affect expression of functional, cell surface H-2D^b–epitope V complexes in vitro and/or influence the ability of cells expressing T antigen to elicit an epitope V-specific CTL response in vivo, a series of epitope deletion derivatives of SV40 T antigen was generated by deleting H-2^b-restricted CTL epitopes from T antigen (Fig. 2). It was of interest to determine whether deleting immunodominant H-2D^b-restricted CTL epitopes from T antigen would result in an increase in the presentation of epitope V by H-2D^b class I molecules.

Cell lines which expressed T-antigen constructs deleted of defined T-antigen CTL epitopes were compared in lysis assays with the SV40-specific CTL clones K-11, Y-1, Y-2, Y-4, and Y-5. As expected, cell lines expressing T-antigen derivatives deleted of defined CTL epitope sequences were not lysed by CTL clones specific for the corresponding epitope(s) (data not shown; see below). Additionally, the results of the ^{51}Cr release assay presented in Fig. 2 illustrate that cell lines which expressed SV40 T-antigen derivatives deleted of epitope I or II/III or both I and II/III did not demonstrate enhanced lysis by the CTL clone Y-5 compared with cells which expressed the intact T antigen. These results imply that the expression of the H-2D^b-restricted CTL epitopes I and II/III does not affect the recognition of epitope V by the CTL clone Y-5.

Effect of insertion of a peptide sequence representing an optimal H-2D^b binding motif on the presentation of H-2D^b-restricted T-antigen CTL epitopes. To further test the hypothesis that cell surface expression of epitope V might be affected by processing and presentation of other stronger epitopes within SV40 T antigen, derivatives of T antigen were generated by the insertion of a peptide sequence, SMIKNLEYM (D^{b}N5), which has been previously characterized as a high-affinity (optimized) binding peptide for the H-2D^b class I molecule (Fig. 3A) (26). In preliminary experiments, synthetic peptides corresponding to T-antigen H-2D^b-restricted epitopes I and II/III and the D^{b}N5 peptide (SMIKNLEYM) were found to stabilize H-2D^b molecules on the surface of RMA/s cells with similar efficiencies (data not shown). One class of T-antigen derivatives was generated by introducing codons specifying the D^{b}N5 sequence and several flanking residues into an SV40 T-antigen-encoding construct which bears an *Eco*RI cleavage site at codon 350 (T350 + D^{b}N5; Fig. 3A). Previous work from our laboratory has shown that this region of SV40 T antigen can be used for expression of heterologous CTL epitopes (25, 62). As a control, a second derivative was generated by insertion of a peptide sequence similar to the D^{b}N5 optimal binding motif but which differed by an Asn-5→Ala substitution (SMIK<u>A</u>LEYM; T350 + d^{b}A5; Fig. 3A). An asparagine residue at position 5 is required for efficient peptide–H-2D^b MHC class I complex formation in vitro (26) and is the residue most frequently found at this position in mixtures of peptides extracted from H-2D^b class I complexes isolated from H-2^b cells (59).

B6 cell lines immortalized by the T-antigen derivatives bearing insertions of the optimal (T350 + D^{b}N5) and Asn-5→Ala-substituted (T350 + d^{b}A5) peptide sequences were compared in a ^{51}Cr release assay for lysis by SV40-specific CTL clones. The results presented in Fig. 3A are representative of several assays in which we failed to detect a significant reduction in the efficiency of CTL lysis of cell lines immortalized by a T-antigen derivative which contained the optimal H-2D^b binding motif. Even lysis by CTL clone Y-5, which in these experiments characteristically lysed wild-type T-antigen-expressing cells at a low efficiency, was not further reduced by the presence of the inserted H-2D^b binding motif.

Similar results were obtained from the analysis of cell lines immortalized by a T-antigen derivative in which the optimal

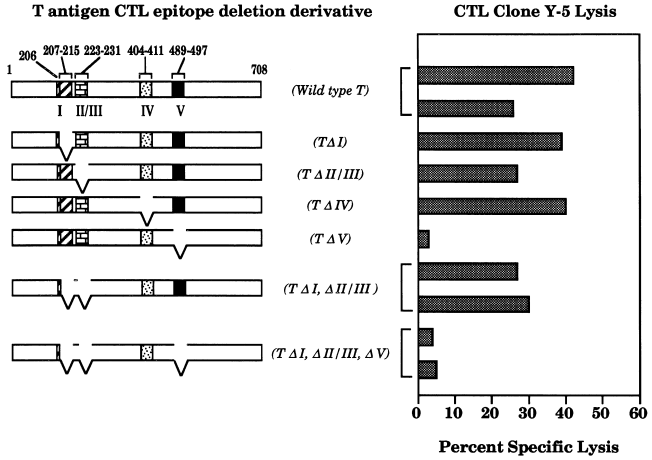

FIG. 2. CTL clone Y-5 lysis of B6 cell lines expressing T-antigen derivatives deleted of various H-2^b-restricted CTL epitopes. T-antigen derivatives generated by deletion of CTL epitope-encoding sequences from SV40 T antigen are illustrated on the left. Epitopes I (206 plus 207 to 215), II/III (223 to 231), IV (404 to 411), and V (489 to 497) are indicated in wild-type T antigen as filled boxes (top). Shortened descriptions used in the text and tables appear in italic. The "Δ" symbol indicates deletion of that epitope. Target cells were reacted with CTL clone Y-5 at an effector-to-target ratio of 10:1 in a 4.5-h ^{51}Cr release assay. Brackets indicate multiple cell lines which express one derivative. Cell lines represented are B6/TpLM234-5Aa and -6Bb, B6/TpLM235-7Ba, B6/TpLM236-8Ab, B6/TpLM237-9Ab, B6/TpLM241-11Bb, B6/TpLM247-14Ab and -14Bb, and B6/TpLM249-15Ab and -15Bb.

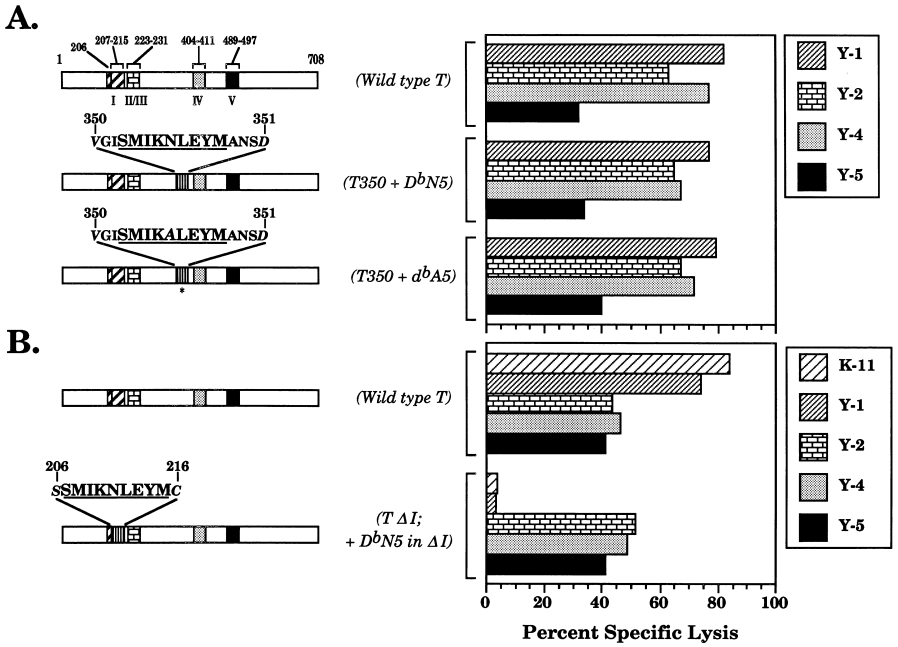

FIG. 3. CTL clone lysis of target cell lines immortalized by T-antigen derivatives bearing insertions of the sequence SMIKNLEYM, which specifies an optimal H-2D^b binding peptide. Relevant T-antigen derivatives are illustrated on the left. Epitopes I, II/III, IV, and V are indicated for wild-type T antigen (top). Shortened descriptions used in the text and tables appear in italic. The filled box representing the SMIKNLEYM 9-mer (D^{b}N5) is indicated. An asterisk is used to indicate an Asn→Ala change at position 5 of the 9-mer, resulting in the sequence SMIKALEYM (d^{b}A5). Residues SMIKNLEYM replace residues 207 to 215 (ΔI) in the lowermost derivative. Amino acid sequences are given for regions of T-antigen derivatives which bear insertions. Proximal native flanking T-antigen residues are indicated in italics. For further details, see Table 1. (A) ^{51}Cr-labeled B6 cell lines expressing the indicated T-antigen derivatives were reacted with SV40-specific CTL clones Y-1, Y-2, Y-4, and Y-5 in a 4.5-h ^{51}Cr release assay. CTL clones Y-1, Y-4, and Y-5 were used at an effector-to-target ratio of 10:1; CTL clone Y-2 was used at an effector-to-target ratio of 20:1. The results represent average lysis values for two (wild-type T; B6/TpLM234-5Aa and -6Bb) or four (T350 + D^{b}N5 and T350 + d^{b}A5; B6/TpTR350N5 and B6/TpTR350A5 clones) cell lines. (B) ^{51}Cr release assay. CTL clones K-11, Y-1, Y-4, and Y-5 were used at an effector-to-target ratio of 10:1; CTL clone Y-2 was used at an effector-to-target ratio of 30:1. Average lysis values are given for two (wild-type T; as in panel A) or four (T ΔI; +D^{b}N5 in ΔI; B6/TpLM301 clones) cell lines.

H-2D^b binding peptide sequence, inserted without additional linker residues, precisely replaced nine T-antigen epitope I residues, 207 to 215. The results of a representative ^{51}Cr release assay in which B6 cell lines which expressed this derivative or the wild-type T antigen were compared are presented in Fig. 3B. As expected, CTL clones K-11 and Y-1, which recognize epitope I, did not lyse cells expressing T-antigen derivatives lacking residues 207 to 215. The results in Fig. 3B further demonstrate that the H-2D^b-restricted CTL clones Y-2 and Y-5 lysed target cells which expressed the T-antigen derivatives in which the optimal H-2D^b binding motif replaced the epitope I residues as efficiently as cells which expressed the wild-type SV40 T antigen.

Induction of SMIKNLEYM-specific CTL in C57BL/6 mice by T-antigen derivatives bearing the corresponding optimal H-2D^b binding peptide sequence. Although previous results from our laboratory support the notion that the protein context surrounding T-antigen residues 350 to 351 allows for efficient processing and presentation of epitopes inserted at that location (25, 62), it was important to show that the D^{b}N5 sequence (SMIKNLEYM), when inserted into T antigen at residue 350 or in place of epitope I, could be processed and presented as an intact epitope in H-2^b cells. To address this question, CTL were generated in B6 mice by immunization and in vitro restimulation with B6 cells expressing T-antigen derivatives bearing the D^{b}N5 or d^{b}A5 sequence. The results presented in Table 4 show that such CTL efficiently lysed target cells pulsed with a synthetic peptide corresponding to the H-2D^b binding motif sequence, SMIKNLEYM (D^{b}N5). The failure of cell line B6/TpTR350A5-41B2 (T350 + d^{b}A5; SMIKALEYM) to elicit a similar response supports the crucial role of asparagine at position 5 in the motif peptide. The results of other experiments confirmed that immunization with the T350 + d^{b}A5 derivative and restimulation with the T350 + D^{b}N5 derivative or immunization with the T350 + D^{b}N5 derivative and restimulation with T350 + d^{b}A5 did not elicit SMIKNLEYM-cross-reactive CTL (data not shown). B6/TpLM301-59A2 cells (T ΔI; +D^{b}N5 in ΔI) express a T-antigen derivative in which residues specifying the 9-mer binding motif replace T-antigen residues 207 to 215 and were also able to induce CTL which recognized targets pulsed with the synthetic peptide SMIKNLEYM (Table 4). The ability of the T350 + D^{b}N5 and the T ΔI; +D^{b}N5 in ΔI cells, which have in common the inserted residues SMIKNLEYM, to elicit CTL which recognize the 9-mer peptide SMIKNLEYM indicates that this 9-mer sequence is an immunogenic CTL epitope and that it can be processed and presented by *H-2^b* cells. Further, the processing and presentation of the SMIKNLEYM peptide by *H-2^b* cells does not reduce presentation of H-2D^b-restricted T-antigen CTL epitopes, including epitope V.

Effect of relocation of epitope V on processing and presentation. The results of several studies suggest that efficient processing and presentation of CTL epitopes requires a permissive protein context. While a CTL epitope may be expressed efficiently from multiple, randomly chosen locations within a protein (17, 29), it has been reported that protein sequences flanking a CTL epitope can prevent efficient processing and presentation (6, 17, 19). We relocated epitope V within T antigen to determine if the immunogenicity of this epitope could be enhanced.

TABLE 4. CTL responses to B6 cell lines immortalized by SV40 T-antigen derivatives containing an optimal H-2D^b binding motif (D^{b}N5, SMIKNLEYM)

Effectors[a]		E:T ratio	% Specific lysis of B6/K-1,4,5 cells pulsed with peptides[b]					
Immunization with B6 cells expressing indicated T antigen	In vitro stimulation		No peptide	LT206–215	LT223–231	LT404–411	LT489–497	SMIKNLEYM
Wild type	T350 + D^{b}N5	50:1	3	50	35	52	3	2
Wild type	Wild type	50:1	2	54	44	85	4	2
T350 + D^{b}N5	T350 + D^{b}N5	50:1	1	62	55	43	2	49
T350 + D^{b}N5	Wild type	50:1	1	55	53	78	2	2
T350 + d^{b}A5	T350 + d^{b}A5	50:1	2	48	35	80	2	3
T350 + d^{b}A5	Wild type	50:1	2	47	39	90	3	2
T ΔI; +D^{b}N5 in ΔI	T ΔI; +D^{b}N5 in ΔI	50:1	3	2	44	66	2	23
T ΔI; +D^{b}N5 in ΔI	Wild type	50:1	1	2	49	85	2	2
	Y-5 (CTL clone)	10:1	−1	0	1	0	45	0

[a] Bulk culture in vitro-restimulated splenocytes prepared from groups of four C57BL/6 mice immunized with T-antigen-immortalized B6 cell lines were used in a 5-h ^{51}Cr release assay at an effector-to-target (E:T) ratio of 50:1. Cell lines used were B6/TpLM234-5Aa (wild type), B6/TpTR350N5-39A1 (T350 + D^{b}N5), B6/TpTR350A5-41B2 (T350 + d^{b}A5), and B6/TpLM301-59A2 (T ΔI; +D^{b}N5 in ΔI). See Table 1.

[b] Chromium-labeled B6/K-1,4,5 cells were pulsed with peptide LT206–215 (epitope I), LT223–231 (epitope II/III), LT404–411 (epitope IV), or LT489–497 (epitope V) or a 9-mer peptide consisting of residues SMIKNLEYM (D^{b}N5).

We constructed T-antigen derivatives in which the locations of epitope V and epitope I were reversed. These experiments were initiated before it was determined that 10 residues specify the optimal epitope I (42). An initial series of epitope exchange derivatives was constructed by replacing nine epitope I residues (207 to 215, AINNYAQKL) in the amino-terminal half of T antigen with nine codons specifying epitope V (489 to 497, QGINNLDNL). Derivatives were also constructed by replacing nine epitope V residues in the carboxy-terminal half of T antigen (489 to 497) with nine epitope I residues (207 to 215). Relevant T-antigen derivatives are illustrated in Fig. 4. B6 fibroblast cell lines expressing these and other relevant derivatives were generated and compared for relative efficiency of lysis by SV40-specific CTL clones in ^{51}Cr release assays. The results of a representative assay in which target cells were reacted with CTL clones Y-1, Y-4, and Y-5 are presented in Fig. 5. CTL clone Y-5 efficiently lysed target cell lines B6/TpLM281-35Ab and -36Ba (Fig. 5c and d), B6/TpLM279-32Ab

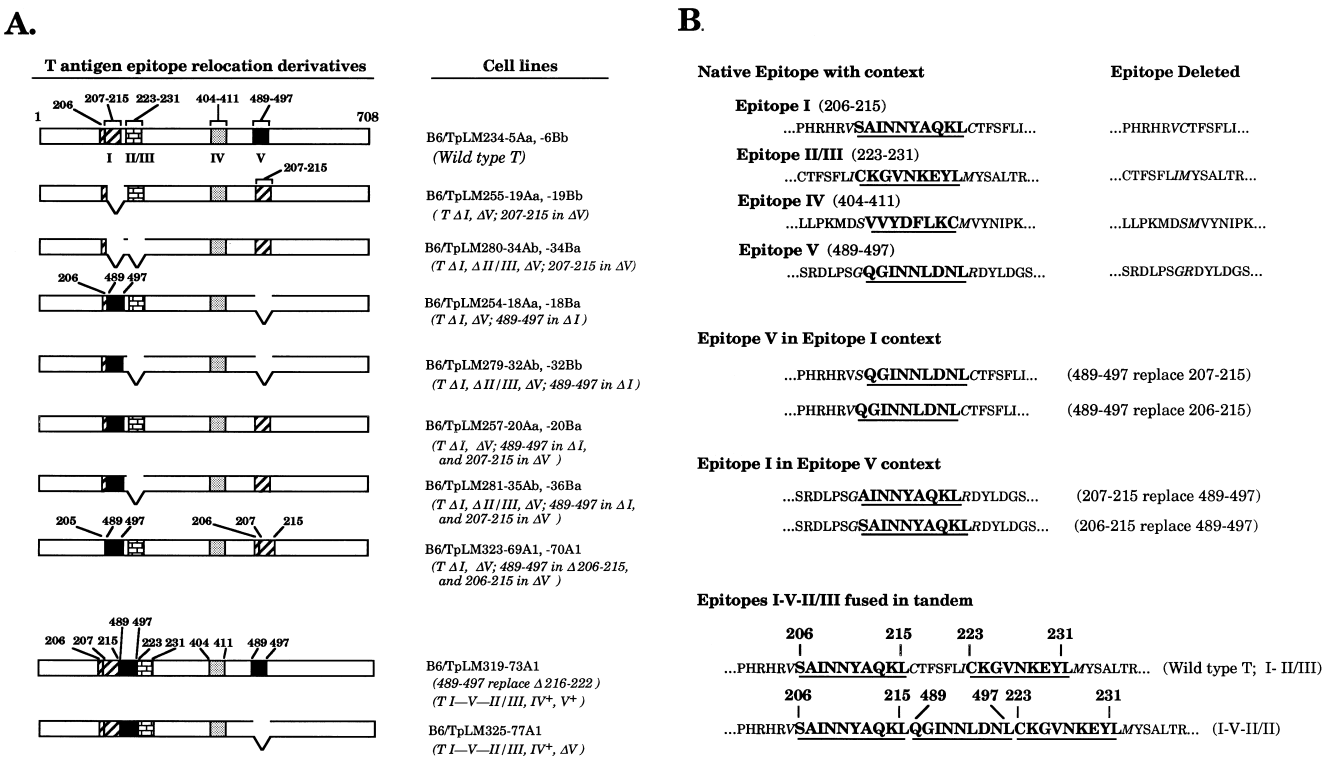

FIG. 4. T-antigen epitope relocation derivatives created by oligonucleotide directed mutagenesis. (A) Diagrams represent the various T-antigen derivatives; corresponding *H-2^b* cell lines are indicated on the right (see also Table 1). Epitopes I (206 plus 207 to 215), II/III (223 to 231), IV (404 to 411), and V (489 to 497) are indicated in the various derivatives by filled boxes and labeled for the wild-type T antigen (top). Epitope deletions are indicated by open spaces above "v" symbols. (B) Predicted coding sequences for regions encompassing and surrounding native or relocated CTL epitopes in SV40 T-antigen derivatives.

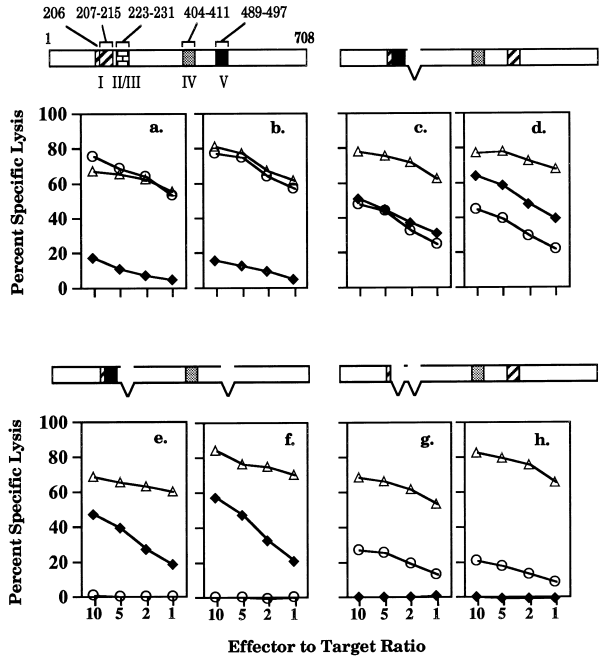

FIG. 5. CTL clone lysis of cell lines expressing T-antigen derivatives in which nine epitope I residues (207 to 215) and nine epitope V residues (489 to 497) have been reciprocally relocated and/or deleted. T-antigen derivatives are described in Fig. 4 and Table 1. Results are derived from a standard 4.5-h ^{51}Cr release assay in which the CTL clones Y-1 (○), Y-4 (△), and Y-5 (◆) were used at effector-to-target ratios ranging from 10:1 to 1:1. Diagrams of the relevant T-antigen derivatives appear above the appropriate panel. Target cell lines: (a) B6/TpLM234-5Aa; (b) B6/TpLM234-6Bb; (c) B6/TpLM281-35Ab; (d) B6/TpLM 281-36Ba; (e) B6/TpLM279-32Ab; (f) B6/TpLM279-32Bb; (g) B6/TpLM280-34Ab; (h) B6/TpLM280-34Ba.

and -32Bb (Fig. 5e and f), B6/TpLM257-20Aa and 20Ba (see below), and B6/TpLM254-18Aa and -18Ba (data not shown); these cells expressed T-antigen derivatives in which epitope V (residues 489 to 497) replaced nine epitope I residues (207 to 215). CTL clone Y-5 also recognized and lysed cell lines which were immortalized by T-antigen derivatives in which epitope V replaced the entire epitope I 10-mer (residues 489 to 497 replace 206 to 215; cell lines B6/TpLM323-69A1 and -70A1; data not shown).

Cell lines such as B6/TpLM280, B6/TpLM257, and B6/TpLM281 which expressed T-antigen derivatives in which nine epitope I residues (207 to 215) replaced nine epitope V residues (489 to 497) were lysed less efficiently by CTL clones K-11 and Y-1 than target cells which expressed the wild-type T antigen (Fig. 5). More recent results indicate that Ser-206 is part of the optimal epitope I peptide (42). Cell lines expressing T-antigen derivatives in which the epitope I 10-mer (SAIN NYAQKL) replaced epitope V (QGINNLDNL) were lysed as efficiently as wild-type T-antigen-expressing cells by the epitope I-specific CTL clones Y-1 and K-11 (data not shown; 51a). Cell lines in which the T-antigen derivatives retained epitopes IV and II/III, respectively, were lysed efficiently by CTL clones Y-4 and Y-2 (Fig. 5 and data not shown).

Turnover of cell surface H-2D^b-epitope V complexes. In another attempt to identify functional differences between epitope V and the immunodominant SV40 T-antigen CTL epitopes, ^{51}Cr release assays were performed with T-antigen-expressing cell lines which had been cultured in the presence of BFA. BFA inhibits protein secretion and thereby blocks presentation of MHC class I complexes containing newly processed viral epitopes (12, 38, 86). The results presented in Fig. 6 illustrate that incubation of cell lines which expressed the wild-type SV40 T antigen (B6/TpLM234-5Aa and -6Bb) in the presence of BFA for 4 h led to a dramatic reduction in lysis by CTL clone Y-5 (Fig. 6a and b). Results obtained in assays using the independently isolated epitope V-specific CTL clones H-1 and B-3 (Fig. 6c and d) confirm that the dramatic BFA-induced reduction of Y-5 target cell lysis is not peculiar to the CTL clone Y-5 and therefore most likely results from a reduction in the abundance of functional epitope V-containing H-2D^b complexes on the surface of BFA-treated targets. This contrasted with a lesser reduction in lysis of the same target cells by the CTL clones Y-1, Y-2, and Y-4 (Fig. 6).

To determine if the protein context surrounding epitope V in T antigen might contribute to the apparent instability observed for H-2D^b–epitope V complexes on BFA-treated targets, cell lines immortalized with T-antigen derivatives in which epitope V was relocated to the epitope I position were used in ^{51}Cr release assays following incubation in the presence of BFA. Y-5-mediated lysis of target cell lines immortalized by T-antigen derivatives in which epitope V had been relocated to an amino-terminal location (B6/TpLM257-20Aa and -20Ba) was rapidly and dramatically reduced following incubation of

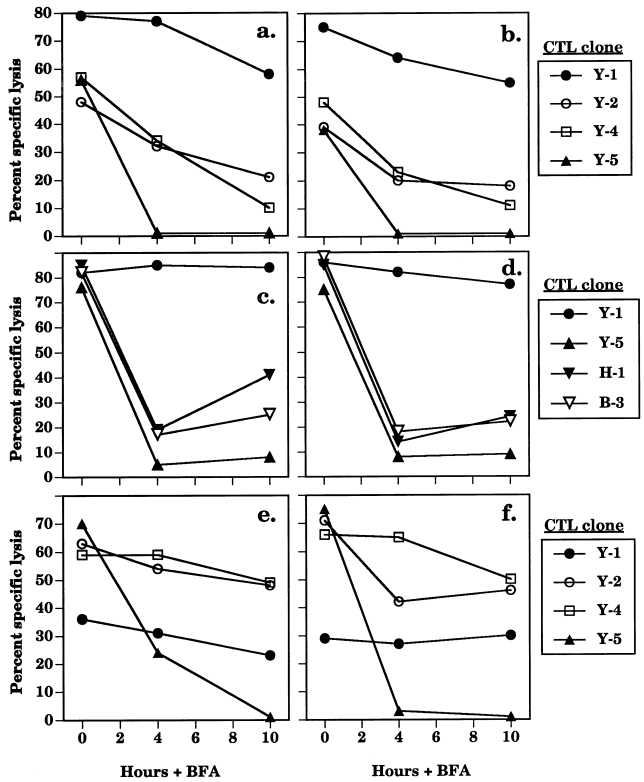

FIG. 6. CTL clone lysis of target cell lines incubated in the presence of BFA. Targets were ^{51}Cr labeled in culture during the 2 h immediately preceding harvest. *H-2^b* cell lines expressing wild-type T antigen (B6/TpLM234-5Aa [a] and B6/TpLM234-6Bb [b]) or a T-antigen derivative in which the locations of residues 207 to 215 and 489 to 497 were reversed (B6/TpLM257-20Aa [e] and B6/TpLM257-20Ba [f]) were incubated in medium containing 4 μg of BFA per ml for either 0, 4, or 10 h prior to harvest and use in a standard 4.5-h ^{51}Cr release assay with the SV40-specific CTL clones Y-1, Y-2, Y-4, and Y-5. CTL clones Y-1, Y-4, and Y-5 were used at an effector-to-target ratio of 10:1, while Y-2 was used at 20:1. Wild-type T-antigen-expressing cell lines B6/TpLM234-5Aa (c) and B6/WT-3 (d) were incubated in the presence of BFA for the indicated times and reacted with the SV40-specific CTL clones Y-1, Y-5, H-1, and B-3 at an effector-to-target ratio of 10:1.

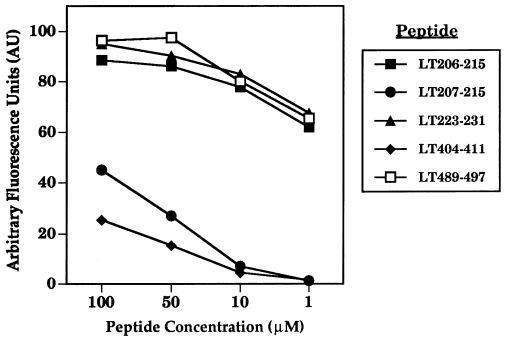

FIG. 7. Stabilization of H-2D^b molecules on the surface of RMA/s cells by synthetic peptides representing SV40 T-antigen H-2^b-restricted CTL epitopes. Peptides correspond to amino acid sequences in T antigen (see Fig. 1). H-2D^b levels were measured by fluorescent flow cytometry following overnight incubation of RMA/s cells in medium containing synthetic peptides at the concentrations indicated. Mean fluorescence intensity is given in arbitrary units. The fluorescence of RMA/s cells incubated without added synthetic peptide was set at zero. RMA cells incubated without added peptide registered as 100 arbitrary units.

targets in the presence of BFA for 4 h (Fig. 6e and f). These results demonstrate that relocation of epitope V within T antigen did not enhance the apparent stability of functional cell surface epitope V–H-2D^b complexes.

Rapid decay of epitope V-MHC class I complexes might be expected if the epitope V peptide bound weakly to H-2D^b molecules. To examine this hypothesis, synthetic peptides representing T-antigen H-2^b-restricted CTL epitopes were compared for relative stabilization of H-2D^b molecules on the surface of TAP2-deficient RMA/s cells. The results presented in Fig. 7 show that peptide LT489-497, which corresponds to the epitope recognized by CTL clones Y-5, H-1, and B-3, stabilized H-2D^b class I molecules on the surface of RMA/s cells as well as did peptide LT206-215 (epitope I) or LT223-231 (epitope II/III). As expected, peptides LT207-215 (N-terminal-deficient epitope I 9-mer) and LT404-411 (epitope IV; H-2K^b restricted) did not efficiently stabilize H-2D^b molecules when used at similar concentrations (Fig. 7) (42). These results suggest that the epitope V peptide does not bind poorly to H-2D^b molecules and imply that other factors most likely contribute to the apparent instability of epitope V–H-2D^b complexes on BFA-treated cells.

CTL induction by cell lines expressing T-antigen epitope deletion and/or epitope V relocation derivatives. The results presented above suggest that processing and presentation of epitope V to CTL clones in vitro is not reduced by the presence of other immunodominant H-2D^b-restricted CTL epitopes (I, II/III, or D^{b}N5 9-mer SMIKNLEYM) in T antigen. It was important to determine whether deleting other CTL epitopes from T antigen or altering the protein context of epitope V within T antigen might affect CTL induction in vivo. C57BL/6 mice were immunized by intraperitoneal injection with cell lines which expressed selected T-antigen derivatives, and splenic lymphocytes were stimulated in vitro with gamma-irradiated cell lines which expressed defined combinations of the immunodominant SV40 T-antigen CTL epitopes and epitope V. B6/WT-19 cells, which express all T-antigen CTL epitopes, or B6/K-3,1,4 cells, which express only epitope V, were used to stimulate lymphocytes recovered from all of the animals so as to minimize variation during the in vitro stimulation phase. Epitope-specific CTL activity was assayed in ^{51}Cr release assays using peptide-pulsed B6/K-1,4,5 cells as targets so that lytic activity directed against the various SV40 T-antigen H-2^b-restricted epitopes could be monitored individually. As a control, peptide-pulsed targets were reacted with SV40-specific CTL clones (data not shown).

The results presented in Table 5 indicate that epitope V, when substituted for epitope I residues 207 to 215 (cell lines B6/TpLM279-32Bb and B6/TpLM254-18Ba; Fig. 4), did not elicit a detectable CTL response in *H-2^b* mice. T-antigen derivatives in which epitope V (residues 489 to 497) replaced 10 epitope I residues (206 to 215) were also unable to elicit detectable levels of epitope V-specific CTL (e.g., B6/TpLM323-69A1 in Fig. 4; data not shown). The results in Table 5 also confirm that CTL responses to epitope I, II/III, or IV were induced when the respective CTL epitope was expressed in cells used to immunize (in vivo) and stimulate (in vitro).

TABLE 5. CTL induction by B6 cell lines immortalized by SV40 T-antigen epitope deletion and exchange derivatives

Effectors[a]		% Specific lysis of B6/K-1,4,5 cells pulsed with peptides[b]				
Immunization with B6 cells expressing indicated T antigen	In vitro stimulation	No peptide	LT206–215	LT223–231	LT404–411	LT489–497
Wild type (B6/WT-3)	B6/WT-19	1	50	27	70	1
	B6/K-3,1,4	4	3	5	6	7
T ΔI-II/III, IV$^-$ (B6/K-3,1,4)	B6/WT-19	1	1	2	2	14
	B6/K-3,1,4	10	9	12	14	32
Wild type (B6/TpLM234-5Aa)	B6/WT-19	1	30	28	66	−1
	B6/K-3,1,4	4	4	5	6	5
T ΔI, ΔII/III (B6/TpLM247-14Ab)	B6/WT-19	1	1	4	83	−1
	B6/K-3,1,4	8	8	6	9	7
T ΔIV (B6/TpLM237-9Ab)	B6/WT-19	3	79	76	5	0
	B6/K-3,1,4	13	13	13	13	12
T ΔI, ΔII/III, ΔV; V in I (B6/TpLM279-32Bb)	B6/WT-19	−1	−1	4	69	0
	B6/K-3,1,4	14	13	16	17	15
T ΔI, ΔV; V in I (B6/TpLM254-18Ba)	B6/WT-19	0	0	38	80	1
	B6/K-3,1,4	11	10	14	13	13

[a] Splenic lymphocytes prepared from groups of four C57BL/6 mice immunized with B6 cell lines expressing the indicated T-antigen derivatives were stimulated in vitro with gamma-irradiated B6/WT-19 and B6/K-3,1,4 cells and reacted with targets at an effector-to-target ratio of 50:1 in a 5-h ^{51}Cr release assay. Names of cell lines used for immunization appear in parentheses after the T-antigen description. See Table 1 and Fig. 4.

[b] ^{51}Cr-labeled B6/K-1,4,5 cells were pulsed in RPMI medium containing peptides LT206–215 (epitope I), LT223–231 (epitope II/III), LT404–411 (epitope IV), and LT489–497 (epitope V) at a concentration of 1 μM or with medium containing no peptide.

We examined whether increasing the number of copies of epitope V within a T-antigen derivative might allow for induction of epitope V-specific CTL. B6/TpLM319-73A1 cells express a T-antigen derivative in which a second copy of epitope V (residues 489 to 497) replaces the seven residues which separate epitopes I and II/III in the wild-type T antigen (Fig. 4). B6/TpLM325-77A1 cells express a corresponding T-antigen derivative from which residues 489 to 497 (native epitope V location) had been deleted (Fig. 4). Both B6/TpLM319-73A1 cells and B6/TpLM325-77A1 cells were recognized efficiently by CTL clone Y-5 (data not shown). B6/TpLM319-73A1 cells which contain two copies of epitope V were unable to elicit a detectable epitope V-specific CTL response in *H-2*^{b} mice (data not shown).

DISCUSSION

While much has been learned about factors which control immunodominance for MHC class II-restricted T cell epitopes (for a review, see reference 64), relatively little is known about the factors which modulate the immunogenicity of MHC class I-restricted CTL epitopes. Results presented here demonstrate that an immunological hierarchy does exist between the multiple H-2^b-restricted CTL epitopes within SV40 T antigen. In cytotoxicity assays utilizing bulk culture CTL, SV40 T-antigen CTL epitopes I, II/III, and IV behave as coimmunodominant CTL epitopes in C57BL/6 mice in that CTL specific for each of these epitopes are readily demonstrated following immunization of C57BL/6 mice with syngeneic SV40 T-antigen-expressing cell lines. By contrast, epitope V appears to be subdominant or immunorecessive within the context of T antigen. That is, epitope V-specific CTL were not detected in bulk culture CTL derived from B6 mice immunized with syngeneic cells expressing T-antigen derivatives which contained an intact copy of at least one other T-antigen CTL epitope. Estimations of CTLp frequencies reveal that CTL directed against epitope IV predominate in C57BL/6 (*H-2*^{b}) mice immunized with syngeneic cells expressing the full-length T antigen. Under the same conditions, CTLp specific for epitopes I and II/III are detected at frequencies that are similar to each other but 5- to 10-fold lower than the frequency of epitope IV-specific CTLp. Epitope V-specific CTLp were not detected following immunization with the intact T antigen.

To investigate mechanisms which may contribute to the recessive nature of epitope V, genetically altered SV40 T-antigen derivatives were constructed, used to generate immortalized *H-2*^{b} cell lines, and examined for recognition by epitope-specific CTL clones and immunogenicity (CTL induction in vivo). The T-antigen derivatives used in this study were specifically altered so that in most cases, it was unnecessary to incorporate additional nonnative flanking or joining residues. We have taken this approach to investigate whether factors contributed by T antigen itself (including its CTL epitopes) might influence the cell surface expression of functional H-2D^b-restricted epitope V complexes on *H-2*^{b}-derived fibroblast cell lines and/or the immunogenicity of epitope V in C57BL/6 mice.

Our results point to several novel and important findings. (i) Processing and presentation of the immunorecessive epitope V to CTL clones in vitro appeared to be unaffected by the presence of other immunodominant H-2D^b-restricted CTL epitopes in T antigen. These results rule out a model whereby epitope V presentation is reduced by competition with other stronger epitopes during T-antigen processing and presentation. (ii) The protein context within T antigen does not in itself determine the immunorecessive nature of epitope V. Epitope V-specific CTL clones lysed cells immortalized by T-antigen derivatives in which residues 489 to 497 had been relocated. Epitope V remained immunorecessive when relocated within T antigen, despite enhanced recognition by the epitope V-specific CTL clone, Y-5. (iii) The presence of two copies of epitope V at separate locations within T antigen was not sufficient to induce an epitope V-specific CTL response in C57BL/6 mice.

Our conclusions rely on several assumptions which remain to be validated by identification of native T-antigen CTL epitope peptides eluted from MHC class I complexes isolated from T-antigen-expressing cells. For example, on the basis of available evidence, we expect that the native epitope V peptide will include T-antigen residues 489 to 497 (QGINNLDNL). Results presented here support our previous conclusions that T-antigen residues 489 to 497 represent the immunologically relevant CTL recognition site V of SV40 T antigen. That is, epitope V-specific CTL clones efficiently recognized cell lines which expressed T-antigen derivatives in which residues corresponding the minimal epitope V sequence (15) were relocated to alternate positions within T antigen (Fig. 5 and 6). In experiments now in progress, genetically altered T-antigen derivatives are being used to confirm the identity of the naturally processed peptides which correspond to the CTL recognition epitopes I, II/III, IV, and V.

A recent report (1) has pointed out that T-antigen residues 492 to 500 (NNLDNLRDY) represent another semidominant H-2^b-restricted CTL epitope. In an attempt to confirm those results, we synthesized a peptide corresponding to T-antigen residues 492 to 500. The synthetic peptides LT489-497 (QGI NNLDNL, epitope V) and LT492-500 (NNLDNLRDY) correspond to overlapping sequences in T antigen. We have been unable to detect CTL which lyse targets pulsed with the peptide LT492-500 following immunization of C57BL/6 mice with *H-2*^{b} cell lines expressing T antigen (data not shown). Further, SV40-specific CTL clones (including epitope V-specific CTL clones) fail to lyse targets pulsed with the synthetic peptide LT492-500 (data not shown). It should be pointed out that the mutation which affects the epitope V region in cell line B6/K-1,4,5 and prevents recognition by CTL clone Y-5 specifies a Phe→Ile substitution at position 491 (41). Therefore residue 491 should lie outside of the immunodominant epitope (residues 492 to 500) proposed by Alsheikhly (1). Bulk culture CTL raised against the intact T antigen do not lyse B6/K-1,4,5 cells (Tables 2, 4, and 5).

Previous results from our laboratory had indicated that the immunogenic potential of epitope V may be compromised (at least in part) by the presence within T antigen of three other immunodominant H-2^b-restricted CTL epitopes (I, II/III, and IV). We wanted to determine whether reducing the number of dominant CTL epitopes within T antigen might enhance CTL recognition and/or immunogenicity of epitope V in T antigen. Our results rule out competition between epitope V and the immunodominant H-2D^b-restricted T-antigen CTL epitopes (I and II/III) as the basis for the immunorecessive nature of epitope V. Insertion of another H-2D^b-restricted semidominant CTL epitope which represents an optimized H-2D^b binding motif failed to further reduce target cell lysis by CTL clone Y-5. We note that the relative abundance of T-antigen CTL epitope peptides presented on T-antigen-expressing cell lines remains to be determined. Therefore, our conclusions regarding levels of epitope expression are based on the relative efficiency of target cell lysis by CTL clones as an indicator of relative levels of epitope presentation. CTL lysis efficiency has been correlated with the abundance of cell surface MHC class I epitope peptide containing complexes between appropriate threshold limits (11, 34). We favor a model in which the ap-

parent lack of intracellular competition in our assays indicates that the capacity for epitope V peptide generation (processing) and transport and/or loading of epitope V into MHC I complexes (presentation) may not be limited by the presence of other dominant H-2D^b-restricted CTL epitopes within T antigen.

The factors which directly influence the immunorecessive nature of epitope V are still not known. The epitope V-specific CTL response induced by immunization of *H-2^b* mice with the epitope loss variant cell line B6/K-3,1,4 is weak. It is our working hypothesis that alterations to the T antigen expressed by B6/K-3,1,4 cells allows for the induction of epitope V-specific CTL in vivo. Consistent with this notion, epitope V-specific CTL were not detected following immunization of B6 mice with genetically altered T-antigen derivatives bearing epitope V and two H-2D^b-restricted epitopes (I and II/III) or epitope V and a single H-2K^b-restricted epitope (IV; Table 5). The peptides which correspond to epitopes V and IV are presented by distinct MHC class I proteins (H-2D^b and H-2K^b, respectively) and do not appear to bind to the alternate H-2^b class I molecule (Fig. 7) (42, 50). Results presented here imply that competition between these epitopes for presentation in MHC class I complexes does not occur within living cells. Interference between coimmunodominant T-antigen CTL epitopes and epitope V instead may be exerted indirectly at an intercellular or system level (47, 63). Immunoregulatory signals which dampen the recruitment or proliferation of CTLp directed against dominant CTL epitopes like SV40 T-antigen epitope I, II/III, or IV may prevent proliferation of epitope V-specific CTL. The impact of such autoregulatory signals might be more severe on a weak response like that directed against epitope V (totally squelching it) than on a response induced by dominant CTL epitopes like SV40 T-antigen epitope I, II/III, or IV. In this regard, it will be of interest to establish in vivo functional or regulatory roles for subsets of CD8$^+$ T cells which have been labeled as Th1- and Th2-like on the basis of cytokine secretion profiles (61). Alternately, it will be of interest to determine whether the immunological hierarchy between T-antigen CTL epitopes is maintained in *H-2^b* mice bearing mutations in the genes which encode the CTL regulatory protein Fas (APO-1 or CD95) or Fas ligand (reviewed in reference 13). These proteins are thought to facilitate elimination of autoreactive T lymphocytes by inducing apoptosis but may also aid in limiting vigorous responses to foreign antigens (13).

Our results demonstrate that the immunorecessive nature of epitope V does not result from the effects uniquely contributed by residues which flank the native location of epitope V within T antigen. If so, relocation of epitope V to within the protein context surrounding the dominant epitope I should remove such influences, since the flanking sequences which surround epitope I differ from those which surround epitope V (Fig. 4B). Using in vivo priming assays, we were unable to detect CTL directed against epitope V when it was located in four distinct contexts within T-antigen derivatives which retained at least one other semidominant CTL epitope: (i) within its native location between residues Gly-488 and Arg-498, (ii) between residues Ser-206 and Cys-216, (iii) between residues Val-205 and Cys-216, or (iv) between residues Leu-215 and Cys-223. Cell lines expressing T-antigen derivatives with epitope V relocated to these positions were lysed in vitro by CTL clone Y-5 as well as or better than cell lines which expressed the wild-type T antigen.

The unique and dramatic reduction of Y-5-mediated target cell lysis observed following incubation in the presence of BFA is most likely determined by qualities intrinsic to epitope V and may be a reflection of the stability of H-2D^b-restricted epitope V complexes at the cell surface. This notion is supported by the observation that BFA-treated targets were also lysed inefficiently by two independently isolated epitope V-specific CTL clones, H-1 and B-3. Preliminary reverse transcription-PCR amplification and DNA sequence analysis performed on the T-cell receptor genes transcribed in the epitope V-specific CTL clones Y-5, H-1, and B-3 reveals variable-region conservation in both α and β T-cell receptor subunits but indicates sequence divergence within diversity and joining regions (51). These results suggest that the inhibition of Y-5-mediated target cell lysis following BFA treatment results from a characteristic intrinsic to epitope V rather than being a peculiarity of CTL clone Y-5. These results might imply that the epitope V peptide simply forms less stable H-2D^b complexes than, for example, the epitope I and/or II/III peptides. Alternately, relatively few epitope V–H-2D^b complexes may be expressed on the surface of T-antigen-expressing cells compared with the number of H-2D^b complexes which contain the epitope I and/or II/III peptides. In this case, inhibition of class I exocytosis by BFA might also produce a rapid reduction in Y-5-mediated target cell lysis even if all H-2D^b–T-antigen epitope peptide complexes dissociate at similar rates, because fewer epitope V–H-2D^b complexes were present on the target cells prior to the inhibition of class I exocytosis. In experiments designed to address the relative affinities of T-antigen CTL epitope peptides for class I molecules, a synthetic peptide representing T-antigen residues 489 to 497 (minimal epitope V) was found to stabilize H-2D^b complexes on the surface of RMA/s cells as efficiently as peptides representing the epitope I 10-mer (LT206-215) and/or the epitope II/III 9-mer (LT223-231) (Fig. 7). These measurements were made under equilibrium conditions in which RMA/s cells were incubated overnight in different concentrations of the peptides and analyzed by flow cytometry for class I surface expression following antibody staining. Surface plasmon resonance imaging has been used by others to measure dissociation rate constants for peptide-MHC class I complexes. Chen and coworkers have reported that MHC class I complexes containing peptides representing dominant and subdominant ovalbumin epitopes dissociate at different rates; similar amounts of class I complexes were stabilized on the surface of RMA/s cells following extended incubation in the presence of these peptides (10). It will be of interest to apply this technique to the study of T-antigen CTL epitopes. Our results are consistent with a model wherein qualities determined by the epitope V peptide sequence most likely prevent the accumulation of large numbers of cell surface epitope V–H-2D^b complexes. For example, the epitope V peptide may be degraded readily during antigen processing or may be inefficiently transported by the TAP1/TAP2-dependent transporter. A recent study reports that synthetic peptides corresponding to a subdominant CTL epitope from chicken ovalbumin (Ova 55-62) were more readily degraded in vitro by purified proteasome preparations than peptides corresponding to an immunodominant ovalbumin CTL epitope (Ova 257-264 [54]). The results of another recent study suggest that, at least in permeabilized RMA cells, the efficiencies of TAP-dependent transport of synthetic peptides representing epitope I (206 to 215, SAINNYAQKL) and epitope V (489 to 497, QGINNLDNL) are comparable, while the epitope II/III peptide (223 to 231, CKGVNKEYL) is transported less efficiently (53). Studies are currently in progress to determine if bypassing the TAP1/TAP2 transporter can enhance CTL recognition or immunogenicity of epitope V (60).

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